INSTITUTE REPORT NO. 189

ACUTE ORAL TOXICITY OF 4–NITROPHENYL MONOCHLOROMETHYL (PHENYL) PHOSPHINATE (TA009) IN FEMALE RATS

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DIVISION OF RESEARCH SUPPORT

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PRESIDIO OF SAN FRANCISCO, CALIFORNIA 94129

Acute oral toxicity (LD_{50}) of 4-nitrophenyl monochloromethyl (phenyl) phosphinate (TA009) in female rats (Toxicology Series 52)-- White et al

In conducting the research described in this report, the investigation adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care, Institute of Laboratory Animal Resources, National Research Council.

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Toxicology, Acetylcholinesterase Inhibition, Organophosphinate

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The acute oral toxicity of 4-nitrophenyl monochloromethyl (phenyl) phosphinate was determined in female, albino, Sprague-Dawley rats by using the single dose oral gavage method. LD_1, LD_{50}, and LD_{95} with their 95% confidence limits were calculated by probit analysis. The LD_{50} was 242 mg/kg with the 95% confidence limit (221 mg/kg, 265 mg/kg). The formulation falls in the very toxic range.

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ABSTRACT

The acute oral toxicity of 4-nitrophenyl monochloromethyl (phenyl) phosphinate was determined in female, albino, Sprague-Dawley rats by using the single dose oral gavage method. LD_1, LD_{50}, and LD_{95} with the 95% confidence limit were calculated by probit analysis. The LD_{50} was 242 mg/kg with the 95% confidence limit (221 mg/kg, 265 mg/kg). The formulation falls in the very toxic range.

Key Words: Toxicology, Acetylcholinesterase Inhibition, Organophosphinate

PREFACE

TYPE REPORT: Acute Oral Toxicity (LD_{50}) GLP Study Report

TESTING FACILITY: U.S. Army Medical Research and Development Command
Letterman Army Institute of Research
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Presidio of San Francisco, CA 94129-6800

SPONSOR: U.S. Army Medical Research and Development Command
U.S. Army Institute of Medical Research and Chemical Defense
Aberdeen Proving Ground, MD 21010-5012

PROJECT: 35162772A875 Medical Defense Against Chemical Agents
WU 304 Toxicity Testing of Phosphinate Compounds
APC TL04

GLP STUDY NUMBER: 82033

STUDY DIRECTOR: COL John T. Fruin, DVM, Ph.D., VC
Diplomate, American College of
Veterinary Preventive Medicine

PRINCIPAL INVESTIGATOR: CPT Craig W. White, DVM, VC

CO-PRINCIPAL INVESTIGATOR: SP4 Justo Rodriguez, BS

PATHOLOGIST: MAJ Glen E. Marrs, DVM, MS, VC
Diplomate, American College of
Veterinary Pathologists.

STATISTICIAN: Virginia L. Gildengorin, Ph.D.

REPORT AND DATA MANAGEMENT: A copy of the final report, study protocols, raw data, retired SOPs, and an aliquot of the test compound will be retained in the LAIR Archives.

TEST SUBSTANCE: 4-Nitrophenyl Monochloromethyl (Phenyl) Phosphinate, LAIR Code TA009

INCLUSIVE STUDY DATES: 17 November - 14 December 1982

OBJECTIVE: To determine the acute oral toxicity potential of 4-nitrophenyl monochloromethyl (phenyl) phosphinate in female Sprague-Dawley rats.

ACKNOWLEDGMENTS

The authors wish to thank SP5 Leonard J. Sauers, MS, and SP5 Evelyn M. Zimmerman for assistance in performing this research. A special debt of gratitude is due Claire N. Lieske, US Army Research Institute of Chemical Defense, who provided test compound, continued advice, and willing inter-agency support.

SIGNATURES OF PRINCIPAL SCIENTISTS AND MANAGERS INVOLVED IN THE STUDY:

We, the undersigned, believe the study number 82033 described in this report to be scientifically sound and the results in this report and interpretations to be valid. The study was conducted to comply, to the best of our ability, with the Good Laboratory Practice Regulations outlined by the Food and Drug Administration.

JOHN T. FRUIN / DATE
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Study Director

CRAIG W. WHITE / DATE
CPT, VC
Principal Investigator

GLEN E. MARRS JR. / DATE
MAJ, VC
Pathologist

CAROLYN M. LEWIS, MS / DATE
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Data Manager

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Statistician

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PRESIDIO OF SAN FRANCISCO, CALIFORNIA 94129

REPLY TO
ATTENTION OF:

SGRD-ULZ-QA 26 Jun 84

MEMORANDUM FOR RECORD

SUBJECT: Report of GLP Compliance

I hereby certify that in relation to LAIR GLP study 82033 the following inspections were made:

29 Nov 82

08 Dec 82

14 Dec 82

The report and raw data for this study were audited on 25 Jun 84.

Routine inspections with no adverse findings are reported quarterly, thus these inspections are also included in the 4 Jan 83 report to Management and the Study Director.

NELSON R. POWERS, Ph.D.
DAC
Chief, Quality Assurance Unit

TABLE of CONTENTS

Table of Contents (continued)

Acute Oral Toxicity (LD_{50}) of 4-Nitrophenyl Monochloromethyl (Phenyl) Phosphinate (TA009) in Female Rats (Toxicology Series 52)--White et al

One mission of the US Army Medical Research and Development Command is to develop compounds for prophylaxis against organophosphate intoxication. The organophosphinate class of chemical compounds are promising candidates in this effort. It is hoped that a compound can be found with relatively minor side-effects at doses required to provide significant systemic protection. The phosphinates represent a strategy of prophylaxis whereby a critical percentage of the available acetylcholinesterase is protected from chemical agent by binding with a compound, such as 4-nitrophenyl monochloromethyl (phenyl) phosphinate, from which the enzyme may be reactivated by using standard antidotal therapy (1-4).

Objective of the Study

The objective of this study was to determine the acute oral toxicity of 4-nitrophenyl monochloromethyl (phenyl) phosphinate in female Sprague-Dawley rats.

MATERIALS

Test Substance

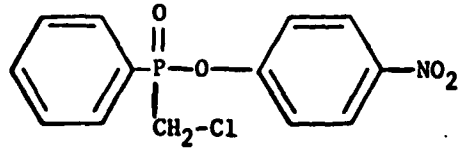
Chemical name: 4-Nitrophenyl Monochloromethyl (Phenyl) Phosphinate

LAIR Code: TA009

Code Name: MCP, CMP

Chemical Abstract Service Registry Number: None known.

Chemical structure:



Empirical formula: $C_{13}H_{11}NO_4ClP$

The test compound was received from the US Army Medical Institute of Chemical Defense, Aberdeen Proving Ground, MD 21010 on 23 June 1982. The test chemical was stored at refrigeration temperature (as suggested by the sponsor) to the time of compounding with the vehicle just before dosing. Detailed chemical data on the test compound is given in Appendix A.

Vehicle

Since phosphinates hydrolyze readily in aqueous solutions, a vehicle which would minimize the rate of hydrolysis was required. The vehicle chosen was a mixture of Tween 80™ (Fisher Scientific Company, Fairlawn, NJ) ethanol and citrate buffer (pH 2.9). Additional information on the vehicle composition is given in Appendix A.

Animal Data

Fifty-one female, albino, Sprague-Dawley rats from Bantin-Kingman Laboratories, Inc., Fremont, CA, were used in this study. To identify each animal individually, ear tags numbered 82D00921 to 82D00973 (with exclusions) were used. The rats' weights on 23 Sep 82 ranged from 146 to 191 g. Additional animal data are given in Appendix B.

Husbandry

The animals used in this study were housed individually in stainless steel, mesh drawer rack cages. No bedding was used in any of the cages.

Diet consisted of Certified Purina Rodent Chow #5002 (Ralston Purina, Checkerboard Square, St. Louis, MO) ad libitum. Water was provided with automatic Lixit dispensers.

The temperature maintained throughout this study was 26 $\pm$ 2°C with a relative humidity of 40 $\pm$ 5%. The photoperiod was 15 hours of light daily (0500 - 2000 hours).

METHODS

Group Assignment/Acclimation

The Beckman TOXSYS Animal Allocation Program was used to assign seven females to each of seven study groups. This program incorporates a weight-biased stratification procedure for allocating the aniamls to the various study groups.

The animals were acclimated for 12 days before dosing. During the acclimation period the animals were observed daily for signs of illness.

Dose Levels

Since the Approximate Lethal Dose (ALD) study indicated that the LD_{50} would be between 200 and 300 mg/kg, doses of 100, 150, 200, 250, and 300 mg/kg were selected for the LD_{50} determination. The amount of dosing solution each animal received was based upon the animal's weight, the desired dose level, and the compound concentration in solution. The dose level was increased volumetrically rather than by varying concentration. The volume administered ranged from 0.90 ml to 3.23 ml. The cage control group was untreated. The vehicle group received 1.5 ml of the vehicle. The dosing was by oral gavage.

All animals were dosed between 0920 and 1050 hours, on 29 November 1982. Sterile, disposable syringes (Becton, Dickinson & Co., Rutherford, NJ) fitted with 16-gauge, 3-inch, ball-tipped feeding tubes (Popper & Sons, Inc., New Hyde Park, NY) were utilized. The dosing procedures were conducted without animal sedation or anesthesia.

Compound Preparation

A 2.0 percent phosphinate solution was prepared as described in Appendix A. The test compound solution was prepared within one hour before dosing. Results of hydrolysis measurements of the dosing solution performed immediately after preparation and within 30 minutes after administration are given in Appendix A.

Test Procedures

The animals were observed on the day of dosing for mortality and signs of acute toxicity throughout the dosing procedure, and at 1200 and 1600 hours. Observations were conducted daily for the remainder of the study. Body weights were recorded just before dosing and twice weekly until death or study completion. Appendix C contains a complete listing of observation periods.

All animals assigned to this study were subjected to a complete gross necropsy. Animals which survived the entire study period underwent necropsy immediately after sacrifice by barbituate overdose.

Statistical analyses were performed on the study data. The LD_1, LD_{50}, and LD_{95} were derived by Bliss probit analysis, as described[1] by Finney (5). The program, PROBIT, written for the Data General Model C330 Computer, was used to determine the probit curve and lethal dose values. The statistician's report appears in Appendix D.

The dosing phase of this study was accomplished according to the protocol and applicable amendments. Groups 5, 6, and 7 were divided into two doses. Double dosing of these groups was required in an attempt to prevent reflux of the calculated dose. The second dose was delivered within one hour of the first.

Raw Data and Final Report Storage

A copy of the final report, study protocols, r . data, retired SOPs and aliquot of the test compound will be retained in the LAIR Archives.

RESULTS

Mortality

Table 1 lists the compound-related deaths by group and the percent mortality.

Table 1

Compound-Related Deaths by Group

Group	Dose Level	Compound-Related Death/ Number in Group	Percent Mortality
1	Untreated Controls	0/7	0
2	Vehicle Controls	0/7	0
3	100 mg/kg	0/7	0
4	150 mg/kg	0/7	0
5	200 mg/kg	1/7	14
6	250 mg/kg	4/7	57
7	300 mg/kg	2/7*	29

*Lower than predicted mortality due to regurgitation of the dosing material within 30 minutes after dosing.

Lethal Dose Calculations

The computer-generated lethal dose values for selected percentages of the population are presented in Table 2. Data from Dose Group 7 was not included in the probit analysis determination since the dose these animals actually received was considerably lower than that administered due to excessive refluxing of the dosing solution.

Table 2*

Lethal Dose (LD) Levels in TA009 in Female Rats

Percent Population	Lethal Dose (mg/kg)	95% Confidence Limit (mg/kg)
LD_1	163	(89, 297)
LD_{50}	242	(221, 265)
LD_{95}	320	(172, 596)

*Statistician Report (Appendix D)

Clinical Observations

The first signs of drug toxicity were retching and salivation. The saliva was extremely frothy with a distinct yellow discoloration which stained the coat. Slight to moderate depression of the pull and pinch reflex was observed in Dose Groups 4, 5, 6, and 7. Only one animal in Dose Group 4 developed the reflex depression. Inactivity, sluggishness, equilibrium disturbances with a loss of gait and staggering were the primary toxic signs observed in animals that were terminated after the 14-day observation period. Increased depth of respiration and a decreased rate were observed frequently, especially in Groups 5, 6, and 7. Increased respiratory rates with decreased depth were also observed, but at a much lower frequency. Rough hair coats, humpback posture, and depression of the righting reflex were also noted in some animals. Diarrhea was observed from animals in Dose Groups 6 and 7. The clinical signs were dose-related in intensity and frequency of effect and were correlated with mortality.

Gross Pathological Observations

The mortalities appear to have been caused by the test compound. Animal Number 82D00971 from Group 6 was left inadvertently in the GLP Suite refrigerator for four days after death. This animal was not submitted for gross necropsy. The cause of death in the animal that was not submitted to necropsy appears to have been the test compound, so the animal was not removed from the study. The pathologist's report is included as Appendix E.

DISCUSSION

The calculated LD_{50} for 4-nitrophenyl monochloromethyl (phenyl) phosphinate in female Sprague-Dawley rats was 242 mg/kg with a 95 percent confidence limit (221 mg/kg, 265 mg/kg). The LD_{50} is within the very toxic range (6).

Clinical signs of toxicity included depression, inactivity, ataxia, loss of equilibrium and gait, decreased respiratory rate, decreased respiratory depth, rough hair coat, humpback posture, salivation, retching with reflux of dosing material, and death.

A plateau of lethality occurred with increasing dose levels. This was attributed to the inability of animals in Dose Group 7 to retain the entire dose administered because of retching and excessive salivation. Salivation and retching are parasympathetic manifestations of the cholinesterase inhibition produced by phosphinate compounds. Although no chemical analyses were performed to confirm that the saliva and refluxed material contained 4-nitrophenyl monochloromethyl (phenyl) phosphinate, its hydrolysate, p-nitrophenol, is the same bright yellow color as the material observed in the saliva.

CONCLUSION

The LD_{50} for 4-nitrophenyl monochloromethyl (phenyl) phosphinate (TA009) was determined to be 242 mg/kg in female Sprague-Dawley rats. The formulation is considered very toxic (6).

RECOMMENDATION

4-Nitrophenyl monochloromethyl (phenyl) phosphinate (TA009) should be considered for further safety testing for eventual human use, provided efficacy is verified.

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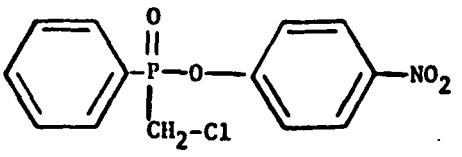
Page

APPENDICES

CHEMICAL DATA

Chemical name: 4-Nitrophenyl Monochloromethyl (Phenyl) Phosphinate

Structural formula:



Empirical formula: $C_{13}H_{11}ClNO_4P$

pH: N/A non-aqueous

Physical state: White crystalline solid

Boiling point: N/A

Melting point: 77-78.5 C

Stability: Dr. Lieske (Biomedical Laboratory, Aberdeen Proving Ground, Aberdeen, MD 21005) indicated the compound would remain stable for two years if refrigerated.

Name of contaminants and percentages: unknown

Manufacturer: Ash Stevens
Detroit Research Park
5861 John C. Lodge Freeway
Detroit, MI 48202

Manufacturer Lot Number: MP-07-201
The test compound was stored in a freezer with no exposure to light.

Dosing Solution: 4-nitrophenyl monochloromethyl (phenyl) phosphinate formulated with Tween 80™, EtOH, and citrate buffer (LAIR SOP-OP-STX-45, Preparation of Compounds Unstable in Water for SLRL Assay).

A 2.0 percent phosphinate solution was prepared with 1.5 g 4-nitrophenyl monochloromethyl (phenyl) phosphinate. 15.0 ml Tween 80™, 7.5 ml (100 %) ethanol, 52.5 ml citrate buffer (50 mM) at a pH of 2.9. The vehicle was the same as above without phosphinate.

pH: 2.9

Physical state: liquid/clear yellow

Boiling point: N/A

Melting point: N/A

Compound refractory: N/A

Contaminants (percentages): Not available

Analysis of Dosing Solution for Hydrolysis:

The phosphinate solution and vehicle were assayed for intact and hydrolyzed phosphinate immediately after preparation and dosing. P-nitrophenol, a product of phosphinate hydrolysis, was quantitated spectrophotometrically at 400 nm using a value of 18,300 for the molar extinction coefficient. Absorbance was measured with a Gilford 2400-S Spectrophotometer in accordance with LAIR SOP-OP-STX-49 "Spectophotometric Measurement of P-nitrophenol for Phosphinate Determination". The concentration of unhydrolyzed phosphinate in the dosing solution was determined from the difference in p-nitrophenol concentration before and after NaOH hydrolysis. The initial hydrolyzed phosphinate was divided by the total hydrolyzed phosphinate to obtain the percent hydrolysis for each solution. The "predosing" measurements of hydrolysis were less than 7% while the "after dosing" measurements were less than 15%. Hydrolysis of the phosphinate solution during dosing averaged 6%.

ANIMAL DATA

Species: Rattus norvegicus (albino laboratory rat)

Strain: Sprague-Dawley

Source: Banton Kingman
Fremont, CA

Sex: Female

Date of Birth: 7 October 1982

Method of Randomization: RANDOM Computer Program; SOP OP-ISG-21

Animals in each group: 7 female animals

Condition of animals at start of study: Normal

Body weight range at dosing: 173 - 213 grams

Identification Procedures: Ear tagging procedure (SOP OP-ARG-1) tag numbers between 82D00921 - 82D00973 with exclusions.

Pretest conditioning: Quarantine/acclimation 17-28 November 1982

Justification: The laboratory rat has been proven to be a sensitive and reliable system for lethal dose determination.

HISTORICAL LISTING OF STUDY EVENTS

Date	Event
17 Nov 82	Fifty-one female Sprague-Dawley rats were received at LAIR. Rats were housed individually and were ear-tagged. Animals were weighed and 2 animals were submitted for quality control necropsy.
22 Nov 82	Animals were randomized, divided into dose groups and weighed.
29 Nov 82	Animals were weighed, dosed, and observed. All animals that died were necropsied.
30 Nov - 13 Dec 82	All animals were observed daily for mortality and clinical signs.
17,19,22,26,29 Nov & 3,6,10,14 Dec 82	All animals weighed.
14 Dec 82	All surviving animals were weighed, sacrificed and necropsied.

STATISTICAL ANALYSIS

Bliss method of probit analysis was used to determine the LD_1, LD_{50}, and LD_{95} values along with the corresponding 95% confidence limits (Table 2). The program, PROBIT, was used to determine the probit curve and the lethal dose values. The probit regression line fit to the data was:

$$Y = -27.2 + 13.5 \log X$$

where X is the dose and Y the corresponding probit value.

Virginia L. Gildengorin

VIRGINIA L. GILDENGORIN, Ph.D
DAC, Statistician
Date:

APPENDIX D

PATHOLOGY REPORT

GLP Study 82033

Oral Lethal Dose (LD_{50}) Test in Female Rats of 4-Nitrophenyl Monochloromethyl (Phenyl) Phosphinate (LAIR Code TA009)

History: The female Sprague-Dawley rats in this study were divided into 7 groups and all groups but the cage controls were dosed with either vehicle or vehicle and TA009 by oral gavage. The dosage levels and numbers of female rats in each group were as follows:

Group 1 (Cage controls) - 7 rats
Group 2 (Vehicle controls) - 7 rats
Group 3 (100 mg/kg) - 7 rats
Group 4 (150 mg/kg) - 7 rats
Group 5 (200 mg/kg) - 7 rats
Group 6 (250 mg/kg) - 7 rats
Group 7 (300 mg/kg) - 7 rats

Five rats died between 16 minutes and 2 hours and 22 minutes after dosing and were necropsied: 1/7 in group 5, 3/6 in group 6, and 1/7 in group 7. A rat in group 6 was found dead more than 4 hours after dosing and was not necropsied because of severe autolysis. Refer to investigators comments for further explanation. All other rats survived until termination of the study, 15 days after dosing.

Gross necropsy findings: The red-brown-black discoloration in one or more lung lobes and/or red oily material in the nares or red fluid around the muzzle in 1/1 rat in group 5 and 2/3 rats in group 6 that died suggest that these deaths were most likely due to the toxic effect of the test material and aspiration of test compound and/or vehicle. The discoloration of lung lobes and material within the nares and around the muzzle was most likely due to the reflux of gastric contents caused by convulsions that occurred prior to death.

One of 3 rats in group 6 and 1/1 rat in group 7 that died, over 2 hours after being dosed, had no respiratory lesions. The absence of lesions suggests that these 2 deaths were due to the affect of the tested compound.

The 2/3 rats in group 6 and 1 rat in group 7 that died more than 2 hours after being dosed had yellow stained perineal hair. This indicates that vehicle and/or test compound or their metabolites were being excreted in the urine.

All of the rats that died had yellow oily material in the digestive tract that was probably test material and vehicle.

central annular fibrous constriction of the spleen, that were considered to be incidental findings.

Summary:

1. One rat in group 6 and 1 rat in group 7 most likely died as a result of TA009 toxicity. Both rats lived over 2 hours after dosing and neither rat had gross lesions.

2. One rat in group 6 died over 2 hours after dosing and had gross pulmonary lesions. The TA009 may have contributed to the rat's death.

3. No test compound related gross lesions were observed in the rats on this study other than those due to aspiration of test compound and vehicle.

Glen E. Marrs, Jr.

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Diplomate, A.C.V.P.
MAJ, VC
Assistant Chief, Pathology Services Group
Division of Research Support

25 February 1983

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